STREPTOLYSIN

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The use of the blood-agar plate for the differentiation of alpha and beta hemolytic streptococci[1] has become general, and its value has been questioned by but few investigators. At the same time, a search of the literature on this subject reveals a distressing lack of uniformity of method and little attempt at standardization. Of 130 papers carefully summarized in the monograph of J. H. Brown,[2] no mention was made of the type of blood used in 29 instances. Human blood was employed in 35 cases, rabbit blood in 28, horse blood in 11, while the remainder of the trials were made with beef, sheep, guinea-pig, hog, dog, or goat blood. In many of the investigations where the species of blood used was named, no mention was made of the concentration of blood, or of the method of preparation of the plates.

It will be admitted that only by standardization of method can a profitable comparison of the results of various investigators be made. The most carefully standardized methods are those of Holman,[3] of Blake,[4] and especially that described in the recent monograph of Brown,[2] which came to hand just before the completion of the work about to be described.

This work has concerned itself almost entirely with the hemolysin of the beta type of streptococcus. It has been considered important to study carefully hemolysin production by this organism on blood accessible to general use, to find out whether variation in suitability exists among these bloods, and finally to determine their optimum concentration for maximum production of hemolysin.

It was apparent at once that such quantitative studies could best be carried out in fluid mediums. Hence the first part of this work is devoted to the investigation of hemolysin production by several strains of the beta streptococcus in a standard broth to which various con-

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[1] Jour. Med. Research, 1915, 31, p. 455.
[2] Monographs of the Rockefeller Institute, 1919, No. 9.
[3] Jour. Med. Research, 1916, 34, p. 377.
[4] Ibid., 1917, 36, p. 99.

centrations of the different serums studied were added. The second part concerns itself with the synthesis of a blood plate of maximal differentiating power, utilizing the quantitative data gleaned in the first part. It is considered superfluous to attempt a historical study of work previously done, since this is to be found in an admirably devised tabular form in Brown's monograph.[2]

Human, rabbit, sheep, and horse blood were used in this investigation. The first three are undoubtedly the most easily obtainable, and the first two have been the most widely used. Sheep blood has been employed by a few investigators but no claims have been made, to our knowledge, of a superiority of this blood over that of the human or rabbit. Horse blood was included because of the excellent results obtained with it by Smith and Brown,[1] and by Brown,[2] on blood-agar plates, and by M'Leod,[5] M'Leod and McNee,[6] von Hellens,[7] and others in the study of soluble hemolysin.

PART I

STREPTOLYSIN IN SERUM BROTH CULTURES

Streptolysin has been chiefly studied in two ways. The first and less frequently used consists in the filtration of ascites or serum broth cultures and the qualitative or quantitative investigation of such filtrates. This method was initiated by Besredka.[8] While streptolysin is undoubtedly filterable, the results from filtration have been uncertain and irregular. Certain makes of filter, such as the Maassen, are far more suitable than others, and even in the case of these, large amounts of hemolysin are lost during filtration.

This difficulty has caused the majority of investigators to employ another method—that of titration of whole cultures. The serum broth, with the organisms contained in it, is added directly to washed or unwashed red blood cells. Such use of whole cultures, while permissible for qualitative tests, is a method of dubious value for careful quantitative work. The reason for this opinion will become clear as the experimental results described below are studied. Two objections to the use of this method, which presented themselves before the experiments were begun, may be mentioned in this place. When dilutions of whole cultures are made, large numbers of organisms are carried over into the culture-red blood cell mixtures. When young cultures are being tested, these organisms will naturally continue to multiply and to secrete hemolysin. Hence, it would be very difficult to determine an end point in time of incubation. For example, a dilution might show a trace of hemolysis or none at the end of two hours at 37 C. Examined two hours later, the red blood cells in this tube might be found completely hemolyzed. Again, the tendency of streptococci to form clumps introduces a variable and unreliable factor into the study of closely graded dilutions of whole cultures.

In view of the objections to the two methods just described it was decided to use a third one. This method is not new. On the other hand it has been

[5] J. Pathol. & Bacteriol., 1912, 16, p. 321.
[6] Ibid., 1912-13, 17, p. 524.
[7] Centralbl. f. Bakt., I, O., 1913, 68, p. 602.
[8] Ann. de L'Inst. Pasteur., 1901, 15, p. 880.

very rarely used in the study of streptolysin. In our hands it has proved very satisfactory, and it certainly avoids the errors to which the first two are subject. It consists simply in the use of the supernatants of cultures centrifugated at high speed, i. e., 8,000 revolutions a minute. Such supernatants, while not sterile, contain minimal numbers of organisms. By their use quantitative results, suprisingly accurate and capable of exact reduplication, can be obtained.

The work outlined below had its inception in the observation that the hemolytic zones surrounding colonies of beta hemolytic streptococcus were much wider and clearer on 10% sheep blood-agar plates than on those prepared from similar concentrations of rabbit and human blood. The preliminary hypothesis as to the cause of this superiority of sheep blood involved the idea of a possibly greater susceptibility of the red corpuscles of the sheep to the streptolysin. To test this idea experimentally an attempt was at once made to obtain free hemolysin by the method of M'Leod, slightly modified by us.

The broth used in the work described was made in the same manner throughout. Beef infusion was employed in all cases. Parke, Davis & Co. bacteriologic peptone was added in a concentration of 2%. Normal $NaCO_3$ solution was added till a hydrogen-ion concentration of P_H 7.8 was obtained. The addition of 17-19 c c of alkali per liter was necessary to arrive at this H-ion concentration. The method used for the determination of P_H was that described by Clark and Lubs.[9]

Eighty c c of broth were placed in each of 4 small sterile Erlenmeyer flasks. Fifteen c c of fresh sterile horse serum (57 C. 60′) were added to each flask. These were now seeded with 0.1 c c each of a suspension in 2 c c of sterile NaCl solution, of 1 loop of a 24-hour blood-agar culture of streptococci, strains W-1, M, A-1, and V. The cultures used for seeding were 10% defibrinated rabbit blood-agar slants. The flasks, inoculated with the above strains in the order just mentioned, were labeled 1, 2, 3, and 4, placed in the water bath at 37 C. and incubated for 18 hours. Examination at the end of this time showed a heavy growth of streptococcus in each case. A portion of broth was removed by bulb pipet from each flask and centrifugated at 8,000 revolutions a minute. The clear supernatants were pipetted off and the following dilutions with 0.85% NaCl solution were made for each one.

TABLE 1

ILLUSTRATION OF DILUTION OF SUPERNATANTS FOR TEST OF HEMOLYSIN

Supernatant		NaCl Solution 0.85%	2.5% Suspension Red Blood Cells (3× washed)		
Undiluted	Diluted 1-10		Sheep	Rabbit	
1.0	—	—	0.5	0.5	Final vol. 1.5 c c
—	1.0	—	0.5	0.5	
—	0.8	0.2	0.5	0.5	
—	0.5	0.5	0.5	0.5	Water bath 37 C. 2 hours
—	0.3	0.7	0.5	0.5	
—	0.1	0.9	0.5	0.5	

No hemolysis was observed at the end of two hours in any of the eight series of dilutions made from the four strains. Repeated attempts to dis-

[9] Jour. of Bacteriol., 1917, 2, pp. 1, 109, 191.

cover traces of hemolysin in the supernatants of 18-hour cultures were made. Although we used all of the 6 strains of beta hemolytic streptococcus then at our disposal, these attempts invariably failed.

Besredka,[8] Braun,[10] M'Leod,[5] von Hellens,[7] and others, have laid stress on the instability of streptolysin. Making use of this fact, and of the observation firmly established by Chesney,[11] that broth cultures of various species of bacteria undergo the greater part of their multiplication at a period of from 4-10 or 12 hours' incubation, it occurred to us that the outpouring of hemolysin might be coincident with this logarithmic period of growth, and that when the rapid multiplication stopped, the free hemolysin might rapidly disappear.

A preliminary test of the validity of this hypothesis was made by cutting short the incubation of a serum broth culture of beta streptococcus, strain M, at 8 hours, centrifugating, and testing the supernatant at once against rabbits' and sheep's blood cells.

A small Erlenmeyer flask containing 50 c c of 15% horse serum broth (the serum previously heated to 57 C. for 45 minutes), was seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of 24-hour rabbit blood-agar culture of beta streptococcus M. The culture was incubated in the water bath for 8 hours at 37 C., centrifugated at 8,000 revolutions a minute for 3 minutes, the clear supernatant pipetted off, diluted in 0.85% NaCl solution and tested for the presence of streptolysin against 2.5% suspensions of 3 times washed rabbits' and sheep's red blood cells. The final volume in each tube was 1.5 c c. The tubes were thoroughly shaken, placed in a water bath at 37 C., incubated for 2 hours and readings made.

The degree of hemolysis in the various tubes will hereafter be designated by the following symbols:

C (complete)
C—
++
+
tr. (trace)
tr—
0

The minimal hemolytic dose of any supernatant will be abbreviated as MHD and will be understood as the smallest amount of supernatant giving complete hemolysis of 0.5 c c of a 2.5% suspension of 3 times washed sheep's red blood cells at the end of 2 hours at 37 C. The tubes, thoroughly shaken before incubation, are left undisturbed during the incubation. At the end of the 2 hours at 37 C. the racks are removed from the water bath, and each tube in turn carefully agitated. In tubes in which the reaction has fallen just short of complete hemolysis, a small deposit of red blood cells remains in the bottom. The agitation to which the tubes are subjected brings these cells into suspension. If this suspension is discernible, the tube is read "C —" (complete minus). Only tubes where there is no perceptible turbidity after thorough shaking are read as C (complete). The last tube, reading from low to high dilutions, giving this result, will naturally represent the MHD for the supernatant in question.

[10] Centralbl. f. Bakt., O., 1912, 62, p. 383.
[11] Jour. Exper. Med., 1917, 26, p. 503.

The result of this experiment is summarized in table 2.

TABLE 2

TEST OF SUPERNATANT OF EIGHT HOUR SERUM BROTH CULTURE FOR STREPTOLYSIN

Supernatant Undiluted	Supernatant 1-10	NaCl 0.85	Result (Hemolysis)	
			Sheep's Red Blood Cells 0.5 c c	Rabbit's Red Blood Cells 0.5 c c
1.0	—	—	C	C
—	1.0	—	C	C
—	0.8	0.2	C	C
—	0.5	0.5	C	C—
—	0.3	0.7	++	++
—	0.1	0.9	+	+

Contrasting with the inactivity of the supernatants of 18-hour cultures, this 8-hour supernatant is seen by the rough titration presented in table 2 to be actively hemolytic. The MHD against sheep red blood cells was 0.05 c c, that against the red cells of the rabbit 0.08 c c. It must be remarked here that the titration outlined in table 2 is only roughly quantitative. In experiments made hereafter dilutions between 0.01 and 0.1 c c are invariably graded by hundredths, e. g., 0.01, 0.02, 0.03, etc.

Similar tests were made with various strains of beta hemolytic streptococcus in both horse and sheep serum broth cultures of from 8 to 10 hours' incubation. Supernatants from such cultures were found invariably to be hemolytic. No great difference of resistance of like amounts of the washed red blood cells of the sheep and the rabbit could be discovered. It will be understood that in testing this point one and the same hemolysin was tested against the two kinds of cells, and that the tests were made at the same time. Occasionally a difference such as that noted in table 2 would appear. More often the MHD for the two species of cells would be identical. The same result was found when sheep's red blood cells were compared to those of the human. In this case the variability of result was still less marked, the MHD for the two, tested against the same hemolysin, being the same in every case.

CURVE OF STREPTOLYSIN PRODUCTION IN SERUM-BROTH CULTURES

Before proceeding further in the analysis of the cause of the superiority of sheep blood-agar plates over those made from human and rabbit blood it was considered important to study carefully the time at which a maximal production of hemolysin takes place. Taking the results outlined in the first two experiments into consideration, it should be possible to plot a curve of the appearance, crest, and disappearance of streptolysin from the serum-broth culture.

The broth used in these experiments was, as before, 2% peptone, beef infusion broth, P_H 7.8. The sheep serum employed had been drawn 24 hours previous to the experiment, and had been heated to 57 C. for 45 minutes.

A flask containing 170 c c of broth and 30 c c of heated sheep serum was seeded with 0.1 c c of an 18-hour serum broth culture of beta streptococcus M; 1 c c was removed at once after seeding and incubated for 2 hours at 37 C. with 0.5 c c of 3 times washed sheep red blood cells; 0.5 c c was removed and plated in 10% horse-serum agar for determination of the number of bacteria. The flask was then placed in a water bath at 37 C. and at 2-hour intervals 5 c c were removed under rigid aseptic precautions, centrifugated at

8,000 revolutions a minute for 5 minutes. The supernatants were then pipetted off, iced, diluted in iced 0.85% NaCl solution, and graded dilutions mixed with 0.5 c c of 3 times washed, 2.5% suspension of sheep's red blood cells. All tubes were brought to a final volume of 1.5 c c. The mixtures were incubated for 2 hours at 37 C., readings taken and MHD recorded. By MHD is meant the smallest amount of a supernatant which will completely lake 0.5 c c of a 2.5% suspension of 3 times washed sheep red blood cells. The end point of the reaction was determined in the manner described above.

At intervals identical with the test for hemolytic power of supernatant, small amounts of culture were removed, suitably diluted, and plated in 10% horse-serum agar for determination of the rate of multiplication of the streptococci. The result of the experiment just described is summarized in table 3.

The MHD found at succeeding 2-hour intervals are plotted in figure 1. The abscissae of this graph representing time in hours, and the ordinates the reciprocals of the MHD's. For example, an MHD of 0.04 would be recorded as 25, of 0.02 as 50, and so on.

TABLE 3

CURVE OF HEMOLYSIN PRODUCTION, BETA STREPTOCOCCUS M

Hours at 37 C.	Amount Supernatant	Result	MHD	Hours at 37 C.	Amount Supernatant	Result	MHD
0	1.0	0	—	8 (cont.)	0.01	++	
					0.0075	++	
2	1.0	0	—		0.005	tr.	
	0.1	0					
	0.09	0		10	0.1	C	
	0.08	0			0.09	C	
					0.08	C	
4	1.0	0	—		0.07	C	
	0.1	0			0.06	C	
	0.09	0			0.05	C	
	0.08	0			0.04	C	
	0.07	0			0.03	C	0.03
	0.06	0					
	0.05	0			0.02	C—	
	0.04	0			0.01	++	
	0.03	0			0.0075	tr.	
	0.02	0			0.005	0	
	0.01	0					
				12	0.1	C	
6	1.0	C			0.09	C	
	0.1	C			0.08	C	0.08
	0.09	C					
	0.08	C			0.07	C—	
	0.07	C			0.06	++	
	0.06	C			0.05	++	
	0.05	C			0.04	+	
	0.04	C	0.04		0.03	tr.	
					0.02	0	
	0.03	C—			0.01	0	
	0.02	++			0.0075	0	
	0.01	tr.			0.005	0	
8	0.1	C		14	1.0	+	—
	0.09	C			0.1	0	
	0.08	C			0.09	0	
	0.07	C			0.08	0	
	0.06	C			0.07	0	
	0.05	C			0.06	0	
	0.04	C			0.05	0	
	0.03	C			0.04	0	
	0.02	C	0.02		0.03	0	
					0.02	0	
					0.01	0	

The results of this experiment indicate that the presence of streptolysin in the supernatants of serum-broth cultures is of short duration, and that the crest or maximum amount is attained at a very early period in the life cycle of the culture. The results of the bacterial counts, made coincident with the test for hemolytic power, are also instructive. The "lag" period described by Chesney,[11] was represented by a very slight increase in the number of organisms per c c for the first six hours. Very rapid multiplication then set in and continued through the 8, 10, and 12-hour tests, at which time the number of viable organisms per c c, as evidenced by the bacterial counts, reached its maximum. It would seem, then, from this experiment that the greatest outpouring of hemolysin occurs in the early stages of the logarithmic growth period. When multiplication comes to a standstill the free hemolysin deteriorates very rapidly.

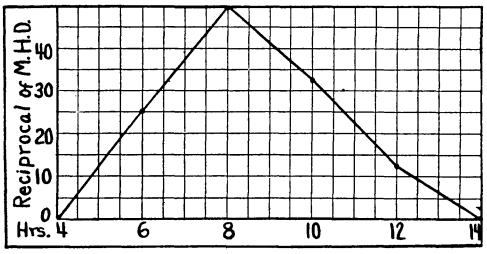

Fig. 1.—Curve of hemolysin production by streptococcus strain M.

Similar determinations of the curve of hemolysin production were made with other strains of beta hemolytic streptococcus. The conditions of these experiments were slightly modified. The sheep serum was used in concentration of 20, instead of 15%, the total volume of fluid in the culture flask was 100 instead of 200 c c, the test flasks were seeded with 9-hour blood-agar cultures instead of 18-hour serum-broth cultures.

Two small Erlenmeyer flasks were prepared, each containing 80 c c of the standard broth and 20 c c of 24-hour old sheep serum heated to 57 C. for 45 minutes. Flask 1 was seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of a 9-hour rabbit blood-agar culture of beta streptococcus A-1. Flask 2 was seeded with a like amount of a similarly made suspension of a 9-hour rabbit blood-agar culture of beta streptococcus V. Both of these flasks were placed in the water bath at 37 C., and at intervals of 2, 4, 5, 6, 7, 8, 9, 10, 12 and 13 hours, 5 c c portions were removed from each flask, centrifuged at 8,000 revolutions a minute for 3 minutes, the supernatant pipetted off, iced, and diluted with iced 0.85% NaCl solution. These were tested against 0.5 c c of a 2.5% suspension of 3 times washed sheep red blood cells. Incu-

bation 2 hours at 37 C., water bath. The test series for amounts 0.1 to 0.01 c c were made from dilution 1 : 10. Those from 0.009 to 0.001 from dilution 1 : 100. All tubes were brought to volume of 1 c c with 0.85% NaCl solution, and 0.5 c c of the red blood cell suspension added at once. The test amounts were closely graded, being separated by hundredths from 0.1 to 0.01 c c, by thousandths from 0.009 to 0.001 c c. An illustration of the method of "set-up" is given in table 4.

TABLE 4

ILLUSTRATION OF ARRANGEMENT OF TEST DILUTIONS IN CURVE OF STREPTOLYSIN PRODUCTION FOR STRAINS A-1 AND V

Tube	Supernatant 1-10	Supernatant 1-100	NaCl 0.85	Actual Amount of Supernatant	
1	0.3	—	0.7	0.03	0.5 c c of 2.5% suspension of 3 times washed sheep's red blood cells added to each tube
2	0.2	—	0.8	0.02	
3	0.1	—	0.9	0.01	
4	—	0.9	0.1	0.009	
5	—	0.8	0.2	0.008	
6	—	0.7	0.3	0.007	
7	—	0.6	0.4	0.006	
8	—	0.5	0.5	0.005	
9	—	0.4	0.6	0.004	
10	—	0.3	0.7	0.003	
11	—	0.2	0.8	0.002	
12	—	0.1	0.9	0.001	

The results of this experiment are summarized in table 5.

TABLE 5

CURVE OF STREPTOLYSIN PRODUCTION, STRAINS A-1 AND V

Hours at 37 C.	Dilutions Tested	MHD	
		Strain A-1	Strain V
2	1.0 to 0.2	0	0
4	0.5 to 0.05	0	0
5	0.5 to 0.05	0	0
6	0.5 to 0.01	0	0
7	0.1 to 0.005	0.03	0.006
8	0.03 to 0.001	0.005	0.008
9	0.05 to 0.001	0.008	0.02
10	0.05 ot 0.005	0.02	0.04
12	1.0 to 0.007	0.1	0
13	1.0 to 0.5	0	0

The results represented in table 5 are plotted in curves in figure 2. The reciprocals of the MHD'S are plotted on the ordinates, the time of incubation in hours on the abscissae. The same phenomenon, rapid rise and early complete disappearance of streptolysin from the supernatants, is seen to occur in the case of strains A-1 and V, as that illustrated for strain M in table 3 and figure 1. The phenomenon for strains A-1 and V has been more closely studied than that for strain M, observations between 4 and 10 hours of incubation being made at one hour rather than 2 hour intervals.

The fulminating nature of the outpouring of hemolysin is especially noticeable in the case of strain V. At the end of six hours' incubation little or no free hemolysin had made its appearance; 0.5 c c of undiluted supernatant failed to cause even a trace of hemolysis, At seven hours it had risen from practically nil to an MHD of 0.006 c c. No larger amount than 0.5 c c was tested at the six-hour period. Assuming that 1.0 c c might have caused complete hemolysis of the usual amount of 0.5 c c of the red blood cell suspension, the hemolysin increased 165 times in strength in the course of an hour. Later curves will show increases still more remarkable than the one just described. It will be noted that the disappearance after a maximum has been reached is also very rapid, though it is not as sudden as the rise.

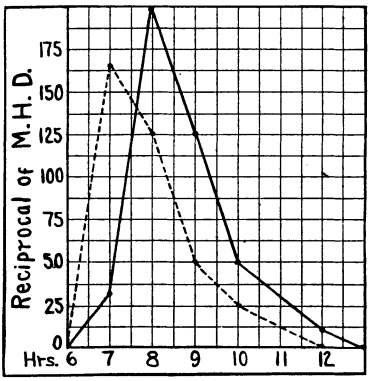

Fig. 2.—Curve of hemolysin production; continuous line, strain A-1; broken line, strain V.

In all three of the curves presented it will be observed that no free hemolysin is demonstrable in supernatants after 14 hours of incubation. On the other hand, M'Leod[5] found 16 to 18-hour serum-broth cultures to contain considerable amounts of the hemolytic principle. Closer examination of the experimental procedure resorted to by this author showed that he seeded his serum-broth flasks with small amounts of 24-hour plain agar cultures. In the experiment just described 9-hour blood-agar cultures were used as source of material for inoculation. This deviation in our technic, resulting from insufficiently close perusal of M'Leod's carefully outlined directions, was thought to be a possible cause of the discrepancy in result. It is to be expected that the older the culture used for seeding, the longer will be the period of lag preceding maximal rate of growth. This might

have as a consequence a delay in the peak of hemolysin production and a corresponding retardation of disappearance.

This supposition was put to experimental test in the following way.

The conditions of experiment laid down by M'Leod were duplicated as nearly as possible. Broth rendered alkaline by the addition of 14 c c of normal Na_2CO_3 per liter was used, instead of the standard broth heretofore employed. Horse serum, heated to 57 C. for 45 minutes, was substituted for the heated sheep serum used in previous experiments. The organism employed in the test was beta streptococcus, strain M. This strain, with all others tested in this research, had been carried for months on 10% rabbit blood-agar slants. Ten days before the experiment about to be described, it was transferred to plain agar slants and underwent 6 transplants on this medium in the ensuing 9 days. The growth obtained on the plain agar was fair in amount, though not nearly as great as that on 12-hour rabbit blood-agar slants.

Two small Erlenmeyer flasks were prepared as follows:

FLASK 1.—40 c c of broth plus 10 c c of horse serum (56 C., 45 minutes), seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of a 24-hour plain agar culture of streptococcus M.

FLASK 2.—40 c c of broth plus 10 c c of horse serum (56 C., 45 minutes), seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of a 12-hour 10% rabbit blood-agar culture of streptococcus M.

Flasks 1 and 2 were incubated in a water bath at 37 C. for 8 hours. At the end of this time 3 c c were removed from each flask, centrifugated at 8,000 revolutions a minute for 3 minutes, the supernatants pipetted off, iced, diluted with iced 0.85% NaCl solution, and graded dilutions tested against 0.5 c c of a 2.5% suspension of 3 times washed sheep's red blood cells. The mixtures had a final volume of 1.5 c c; the readings were taken at the end of 2 hours' incubation at 37 C.

Similar procedure was resorted to at intervals of 2 hours until no more streptolysin was demonstrable in the supernatant from either flask. The result of this experiment is summed up in table 6.

TABLE 6

COMPARATIVE CURVE OF STREPTOLYSIN PRODUCTION IN SERUM BROTH SEEDED WITH 24 HOUR PLAIN AGAR AND 12 HOUR BLOOD-AGAR CULTURES

Hours of Incubation	Dilutions Tested*		MHD	
	Flask 1	Flask 2	Flask 1	Flask 2
8	0.1 to 0.005	0.05 to 0.003	0	0.004
10	0.1 to 0.005	0.05 to 0.001	0.01	0.008
12	0.05 to 0.001	0.2 to 0.01	0.005	0.03
14	0.05 to 0.01	0.2 to 0.01	0.01	0
16	0.1 to 0.005	—	0.03	—
18	0.2 to 0.01	—	0	—
20	0.5 to 0.01	—	0	—

* Dilutions graded by hundredths between 0.1 and 0.01, by thousandths between 0.009 and 0.001.

The results observed in table 6 are plotted in figure 3, the significance of the ordinates and abscissae being the same as in figures 1 and 2. The delay in production, peak and disappearance just predicted is

shown by this experiment actually to occur. The 8-hour test of Flask 1 failed to demonstrate hemolysin in amounts up to 0.1 c c, while in Flask 2 the supernatant had already reached what was in all probability its maximum of streptolysin production — MHD of 0.004. In Flask 1 maximal production was reached at 12 hours — 4 hours after the probable peak in flask 2. In 14 hours no demonstrable lysin was present in 0.25 c c of supernatant of flask 2. On the other hand, flask 1, tested at the same time, showed an MHD of 0.01, and at 16 hours still contained lysin exhibiting a titer of 0.03.

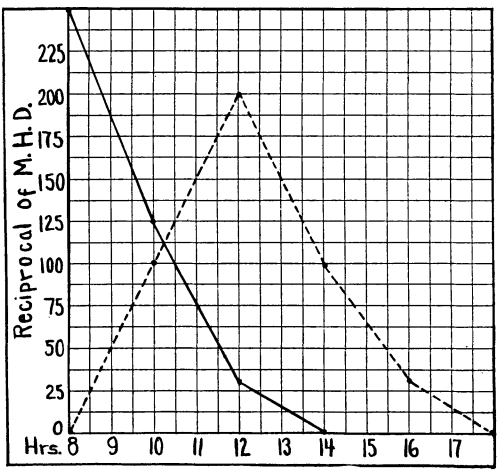

Fig. 3.—Comparative curves of streptolysin production in serum broth seeded with 24-hour plain agar and 12-hour blood-agar cultures of B. streptococcus M. Continuous line, 12-hour blood agar; broken line, 23-hour plain agar.

The persistence of streptolysin at 16 hours as described by M'Leod may be considered to be at least partially explained by the use of older, plain agar cultures for seeding. There still remains the necessity, however, of explaining why many investigators report a persistence of hemolysin not only at 16 to 18 hours, but even for several days.

At the time we first happened on the remarkably fulminating nature of hemolysin production and its correspondingly rapid disappearance from the supernatants of serum broth cultures, we were not aware of the researches made by von Hellens on this subject.[7] Examination of the work of that investigator showed that we were only confirming an observation already made by him. The technic of his experiments differed materially from that of ours, however. Von Hellens did not

use the criterion of complete hemolysis in his quantitative measurements of streptolysin. Instead of this he made colorimetric measurements of amounts of hemoglobin released and reported these findings in terms of percentage of hemolysis. He used at one time 35%, at another 40%; at still others 45, 50, 55, etc. His results are therefore difficult to compare and to correlate. The strictly constant endpoint which is found in the experiments just described, as the smallest amount of supernatant completely hemolyzing a given quantity of red blood cells, is much to be preferred.

In spite of the wide variation of technic it is interesting to note that von Hellens' results correspond exactly to ours in the matter of time of appearance and peak of lysin production. Von Hellens finds that the maximal content of streptolysin appears at from 7 to 8 hours. After this period his results exhibit a marked disagreement with the observations we have just presented. Immediately after the peak has been reached, the lysin in von Hellens' experiments, as in ours, falls off rapidly in amount, but on reaching a certain titer, usually very low, it persists for many hours and even for days. By contrast, the lytic principle has never been demonstrable in our supernatants after 16 hours of incubation, and is rarely to be found after from 12 to 13 hours. It was necessary to investigate the cause of this marked difference.

Many authors have noted that streptolysin appears fairly early in serum-broth cultures. Sekiguchi[12] claims that it may be demonstrated in 3-hour cultures, and that it reaches its maximum in from 15 to 18 hours. M'Leod[5] notes its appearance at from 8 to 10 hours, and thinks that the maximum is reached at from 16 to 18 hours. Braun[10] thinks that the peak of production occurs at 10 hours. No matter what may be the opinion of these workers as to the maximum production time, they all agree that the lysin may persist, though in smaller amounts, for a considerable period of time.

The discrepancy in result is to be found in a difference in technic which has been discussed in the first part of this paper. The writers who have observed this persistence of streptolysin have tested whole cultures. We have been concerned only with supernatants of centrifugates of such cultures. It at once occurred to us that while the actually free hemolysin may have really disappeared from the serum-broth cultures within from 14 to 16 hours, the use of whole, uncentrifugated cultures might still reveal some of the lytic principle operating from the surfaces of the streptococci themselves. It is, indeed, reasonable to suppose that this labile principle might undergo much slower destruction when in contact with the organisms than when free in the

[12] Jour. Infect. Dis., 1917, 21, p. 475.

medium. And since there is every reason to think that streptolysin is a product of secretion of the organisms, it is logical to imagine that a large amount of it is adherent to the cell surfaces.

It was decided to compare experimentally the persistence of the hemolysin in whole cultures and in supernatants of 8,000 revolutions a minute centrifugation, the material for the parallel tests coming, of course, from one and the same flask.

A flask was prepared containing 40 c c of the standard broth described above (meat infusion, 2% peptone, P_H 7.8) and 10 c c of horse serum (56 C., 45 minutes). This was seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of an 8-hour rabbit blood-agar culture of streptococcus *M.* The flask was placed in a water bath at 37 C. and incubated at this temperature for 21 hours. At this point 14 c c were removed from the flask and examined microscopically—there was a growth of streptococcus but no apparent contamination. The portion of 14 c c which had been removed was divided into two equal parts, designated "A" and "B."

A was left at room temperature while B was centrifugated at 8,000 revolutions a minute for 3 minutes. Immediately after the removal of B from the centrifuge A and B were both iced and diluted in iced 0.85% NaCl solution. Graded quantities of each were tested against 0.5 c c of a 2.5% suspension of 3 times washed sheep's red blood cells. The final volume in all tubes was 1.5 c c. Incubation 2 hours at 37 C., water bath.

The results of this experiment are recorded in table 7.

TABLE 7

STREPTOLYSIN CONTENT OF WHOLE CULTURES AND SUPERNATANT AFTER 24 HOURS' INCUBATION AT 37 C.

A (Whole Culture)			B (Supernatant)		
Amount Culture	Result	MHD	Amount Supernatant	Result	MHD
1.0	C		1.0	0	—
0.75	C		0.75	0	
0.5	C		0.5	0	
0.3	C		0.3	0	
0.2	C		0.2	0	
0.1	C		0.1	0	
0.09	C		0.09	0	
0.08	C		0.08	0	
0.07	C		0.07	0	
0.06	C	0.06	0.06	0	
			0.05	0	
0.05	+				

This experiment and others with similar result seem completely to confirm the opinion expressed as to the cause of the persistence of lysin in comparatively old cultures. Lysin is still demonstrable in 21-hour whole cultures, the supernatants of which show no trace of hemolyzing power even in a dose of 1 c c. It seems to us that the only plausible explanation of this difference is to be found in the idea of residual streptolysin operating from the surfaces of the organisms.

Sachs[13] believes that the hemolysin of the streptococcus is almost completely, if not entirely, associated with the germ bodies. Lyall[14] also concludes from certain centrifugation experiments that the streptolysin is to be found in this location. This notion is without doubt erroneous. Free hemolysin does exist and in large quantities, but it must be sought for at a definite time in the life cycle of the culture. When bacterial multiplication is retarded, or stops altogether, the unstable hemolyzing principle rapidly disappears from the supernatant fluids; it may, however, persist for some time on the surfaces of the organisms.

Some observations concerning the hemolysis observed in part A, table 1, are worthy of mention here. The hemolysis in these tubes was very much slower than that ordinarily observed in dilutions of supernatant fluids. In the latter case the reaction invariably runs nearly to completion in from 30 to 45 minutes. Slight changes only occur after this time. In dilutions of from 0.01-0.001, graded by thousandths, an MHD might shift from 0.007 to 0.005 in the time elapsing between 45 minutes and 2 hours, but no greater change than this has been observed to take place after 45 minutes. On the other hand, in the case of the whole culture dilutions recorded in part A, table 7, complete hemolysis had appeared only down to 0.2 c c after 1 hour of incubation. Between 1 and 2 hours the higher dilutions (0.2-0.06) went to completion very gradually. The highest dilution, 0.05 c c, which showed only plus hemolysis at the end of 2 hours of incubation, was completely laked after standing over night on ice. Such marked shifts in readings have never been encountered in the case of supernatants.

Another serious difficulty was encountered in making the readings on the whole culture dilutions (part A). The turbidity due to the large numbers of streptococci in dilutions even as high as 0.05 c c rendered the macroscopic reading of "complete hemolysis" impossible. Microscopic examination of each tube had to be resorted to in order to be sure that this cloud was due to organisms and not to unhemolyzed red blood cells.

It will be understood that when one is dealing with a culture containing many millions of organisms per c c, dilution of 1:100 will leave an enormous number of organisms even in the fractions of a c c which represent amounts of 0.005, 0.004, etc. It was therefore thought

[13] Ztschr. f. Hyg. u. Infekt., 1909, 63, p. 463.
[14] Jour. Med. Research, 1914, 30, pp. 487, 515.

advisable to run parallel curves of lysin content in whole cultures and supernatants in order to determine whether the MHD of the former would far surpass that of the latter. It would appear that the lysin acting from the surface of the organisms in the whole culture dilutions would superpose itself on the free hemolytic principle and so produce a higher MHD. This point was put to experimental test.

A flask containing 200 c c of the standard 20% serum-broth mixture (horse serum, 56 C. 45 minutes), was seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of a 9-hour culture of beta streptococcus strain M. Parallel tests of the whole culture and of supernatants of 8,000 revolutions a minute centrifugates were made. These tests were run 4, 6, 8, 10, 12 and 14 hours after the beginning of incubation. The technic was in every respect the same as that of the test summarized in table 7. Definite amounts were removed from the flask and divided into 2 portions—A was subjected to 8,000 revolutions a minute centrifugation for 3 minutes while B was kept at room temperature during the centrifugation of A. Both A and B were then iced, diluted with iced 0.85% NaCl solution and tested in closely graded dilutions against 0.5 c c amounts of a 2.5% suspension of 3 times washed sheep's red blood cells. The final volume of all tubes was 1.5 c c; incubation 2 hours at 37 C. The results of this test are outlined in table 8.

TABLE 8

COMPARATIVE TITER OF HEMOLYSIN IN WHOLE CULTURE AND SUPERNATANTS

Hours at 37 C.	Amount Tested*		MHD	
	A (Supernatant)	B	A (Supernatant)	B
4	1.0 to 0.2	1.0 to 0.5	0	0
6	0.2 to 0.01	1.0 to 0.01	0.06	0.05
8	0.01 to 0.001	0.01 to 0.0005	0.004	0.004
10	0.05 to 0.0008	0.05 to 0.0008	0.008	0.003
12	0.05 to 0.005	0.05 to 0.003	0.01	0.005
14	0.1 to 0.01	0.05 to 0.005	0.04	0.01

* Dilutions between 1.0 and 0.2 graded by tenths.
Dilutions between 0.2 and 0.01 graded by hundredths.
Dilutions between 0.01 and 0.001 graded by thousandths.
Dilutions between 0.001 and 0.0005 graded by ten thousandths.

Table 8 shows a coincidence of MHD at the 8-hour period. This period of incubation has been found in the great majority of the numerous tests made to represent the peak of lysin production in 20% sheep or horse-serum broth for strain M. On the other hand, the whole culture (B) continues to increase in potency up to 10 hours, showing at this time an MHD of 0.003. At the 10-hour interval the supernatant had dropped to an MHD of 0.008, this decline taking place as it did in all other experiments of a similar nature.

Can it be concluded that the difference of 0.005 in MHD of A and B at the 10-hour interval represents a superposition of the "surface" lysin on that free in the broth? While it is possible that this is so, we do not think that such a conclusion can be safely made. Our doubt on this point is due to the fact that in testing a 10-hour whole

culture one deals with organisms which are still in the logarithmic phase of growth. Those organisms, carried over into the dilutions for the lysin titration, will doubtless continue multiplication during incubation with the red blood cells. This factor, of course, is not present in the dilutions of supernatant (A). It is indeed this uncontrollable factor of continuance of multiplication in the whole culture-red blood cell mixtures which helps to make quantitative tests of whole cultures inaccurate.

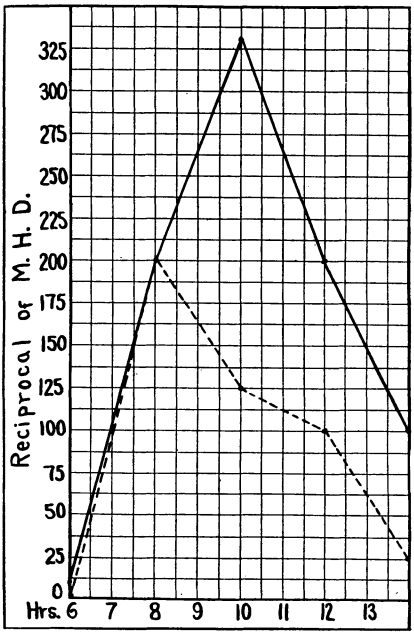

Fig. 4.—Comparative curves of hemolysin in whole cultures and supernatants; B. streptococcus M. Continuous line, whole culture; broken line, supernatant.

At no period in the test was the difference in titer between the whole culture and the supernatant of this culture great. It was greatest at the 10 and 12-hour incubations, being 0.005 at these times. The difference, while not striking, is greater than the experimental error encountered in these tests.

The period at which maximal streptolysin content is present in serum broth has been studied closely. This peak is found to take place in between 7 and 9 hours. The relation of the time of the crest of production and age of the culture used for seeding has been experimentally shown. The relatively short period of persistence of free

streptolysin has been made clear, and in our opinion, a satisfactory explanation of the persistence of this principle in whole cultures has been given. We propose to take up now the amount of streptolysin produced in various concentrations of the serums of different species.

STREPTOLYSIN PRODUCTION IN VARIOUS CONCENTRATIONS OF THE SERUMS OF DIFFERENT SPECIES

The concentration of serum in broth used by the various investigators who have studied hemolysin has differed widely. Besredka[8] used undiluted rabbit serum; M'Leod[5] and M'Leod and McNee[6] employed 15 and 20% horse serum broth. Braun[10] obtained streptolysin from broth which contained 10% rabbit serum. Von Hellens states that 40% horse serum broth is most efficient. Other concentrations of numerous serums have been used by various workers, but it is true that at no time has a careful quantitative comparison of the different media been made. The work outlined below is the result of a careful comparison of sheep, rabbit, human, and horse serums in concentrations ranging from 2.5 to 40%. The majority of authors have employed serum heated to 56 C. in their tests. The comparative efficiency of heated and unheated serum has also been made the subject of study here.

The first experiment attempted concerned itself with a quantitative comparison of streptolysin in 20% sheep, horse, human and rabbit serum broth. The sheep, human and rabbit blood was obtained on the same day. The bloods were defibrinated by rod and the serum obtained by centrifugation. The horse serum used had been standing in the icebox for about one month. All of the serums were simultaneously inactivated by heat of 57 C. for 45 minutes. The following tubes were prepared:

TABLE 9

TUBES PREPARED FOR TEST

	Standard Broth	Serum			
		Sheep	Horse	Human	Rabbit
1	8 c c	2 c c			
2	8 c c	.....	2 c c		
3	8 c c	.....	.....	2 c c	
4	8 c c	.....	.....	.....	2 c c

All tubes were seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of an 18-hour blood-agar culture of streptococcus M. They were placed in the water bath at 37 C. for 8 hours, centrifuged at 8,000 revolutions a minute for 3 minutes, the supernatants pipetted off, iced, diluted with iced 0.85% NaCl solution and the streptolysin determined quantitatively, against 0.5 c c amounts of 3 times washed sheep's red blood cells (2.5% suspension). The technic of making dilutions was in all respects the same as that previously employed. The final volume in all tubes was 1.5 c c incubation—water bath, 37 C. The results of the experiment are correlated in table 10.

Quantities of supernatant from 0.05 to 0.01 c c were made from dilution 1:10 in 0.85% NaCl solution; from 0.01 to 0.001 c c from dilution 1:100 in 0.85% NaCl solution.

The minimal hemolytic dose (MHD) is, as before, the smallest amount of supernatant causing complete hemolysis of 0.5 c c of a 2.5% suspension of washed sheep's red blood cells in 2 hours at 37 C.

TABLE 10

HEMOLYSIN PRODUCTION IN 20% HEATED SHEEP, HORSE, HUMAN AND RABBIT SERUM BROTH

Tube	Amount of Supernatant	Result	MHD	Tube	Amount of Supernatant	Result	MHD
1 (Sheep) Serum	0.05	C		3 (Human) Serum	0.05	C	
	0.04	C			0.04	C	
	0.03	C			0.03	C	
	0.02	C			0.02	C	
	0.01	C			0.01	C	0.01
	0.0075	C					
	0.005	C	0.005		0.0075	C—	
					0.005	++	
	0.0025	++			0.0025	0	
	0.001	+			0.001	0	
2 (Horse) Serum	0.05	C		4 (Rabbit) Serum	0.05	C	
	0.04	C			0.04	C	
	0.03	C			0.03	C	
	0.02	C			0.02	C	0.02
	0.01	C					
	0.0075	C			0.01	C—	
	0.005	C	0.005		0.0075	++	
					0.005	+	
	0.0025	++			0.0025	0	
	0.001	tr.			0.001	0	

The amount of streptolysin produced in 20% sheep and 20% horse serum was practically identical in this experiment. That in the human serum broth titrates about half the amount of the first two, while only one quarter as much could be demonstrated in 20% rabbit serum. Subsequent experiment has shown that the ratio of 4-2-1 for sheep, human, and rabbit serum in 20% concentration is a reasonably constant one. This is especially true of sheep and human. Rabbit serum tends to vary somewhat in its efficiency, the variation usually lying in the direction of a somewhat lower value as compared with the sheep and human serum. Horse serum 20% lies very close to sheep of a like concentration, not only in this, but in many other experiments. If any variation occurs it usually shows the horse serum to be slightly superior to the sheep, e. g., MHD 0.004 for horse as compared with 0.005 for sheep.

The experiment just recorded may be criticized because of the disparity in age between the horse and the other serum. This disparity was eliminated in subsequent tests in which 24-hour old horse serum was used in direct comparison with sheep serum of the same

age. The difference between the two is quantitatively about that mentioned in the preceding paragraph. Fresh heated horse serum is slightly superior to that which has stood for some time. The difference does not appear to be sufficiently marked to make the older material unsuitable for use. The very best titer for streptolysin we have ever obtained was 0.03 (strain M.). This was secured with fresh heated horse serum. The same serum, used two days later, gave a titer of 0.004. Tested two weeks later the same MHD was obtained. Sheep serum in 20% concentration has never yielded a titer of streptolysin higher than 0.005. Consequently, horse serum broth may be said to have a slight but rather constant superiority to sheep serum broth. On the other hand, this titer for sheep serum (0.005), has been observed at least 8 times in 11 attempts. In all except one of these trials the test amounts were graded by thousandths, e. g., 0.007, 0.006, 0.005, 0.004, and so on. The titers attained in the three attempts which did not give MHD, 0.005 for strain M, were 0.0075, 0.01, and 0.02. It is very probable that these variations were due in at least two instances to the use of old broth, and in the other to the employment of a too old culture for seeding (24-hour blood-agar slant). In the latter case an MHD of 0.005 might very well have been obtained had incubation been continued to 10 or 12 hours, instead of being checked at 8 hours.

The next step taken was the systematic test of the four above named species of serums with a view to discovering their optimal concentration in broth for maximal streptolysin production, and to determine the amount of the lysin produced in low concentrations. The latter interested us particularly because the amount of serum in the broth (5%) approximated closely that used in 10% blood-agar plates. It was considered probable that the results of these tests would throw light on the disparity in size and quality of the hemolytic zones on blood-agar plates in which sheep, human and rabbit blood are used. The comparative efficiency of 5, 10, 15, 20, 25 and 30% heated sheep serum broth was first tested. Six tubes were prepared as follows:

TABLE 11

TUBES PREPARED FOR TEST

1.	9.5	c c	standard broth	+	0.5	c c	56 C.	45	minutes	sheep	serum,	(5%)
2.	9.0	c c	standard broth	+	1.0	c c	56 C.	45	minutes	sheep	serum,	(10%)
3.	8.5	c c	standard broth	+	1.5	c c	56 C.	45	minutes	sheep	serum,	(15%)
4.	8.0	c c	standard broth	+	2.0	c c	56 C.	45	minutes	sheep	serum,	(20%)
5.	7.5	c c	standard broth	+	2.5	c c	56 C.	45	minutes	sheep	serum,	(25%)
6.	7.0	c c	standard broth	+	3.0	c c	56 C.	45	minutes	sheep	serum,	(30%)

These were seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of a 16-hour culture of streptococcus V. All the tubes were placed in the water bath for 8 hours at 37 degrees, centrifugated at 8,000 revolutions a minute for 3 minutes, the supernatants pipetted off, iced, and diluted with 0.85% NaCl solution. The dilutions were tested against 0.5 c c of a 2.5% suspension of washed sheep red blood cells. The final volume of all tubes was 1.5 c c. The tubes were thoroughly shaken, incubated in the water bath for 2 hours at 37 C. and the MHD's read at the end of this time. The results of the experiment are summarized in table 12.

TABLE 12

STREPTOLYSIN PRODUCTION IN VARIOUS CONCENTRATIONS OF HEATED SHEEP SERUM BROTH

Tube	Serum Concentrations	Dilutions Tested*	MHD
1	5	0.06 to 0.005	0.04
2	10	0.06 to 0.005	0.03
3	15	0.06 to 0.005	0.01
4	20	0.06 to 0.005	0.005 or >
5	25	0.06 to 0.005	0.02
6	30	0.06 to 0.005	0.02

* Dilutions 0.06 to 0.01 graded by hundredths.
Dilutions 0.01 to 0.005 graded by thousandths.

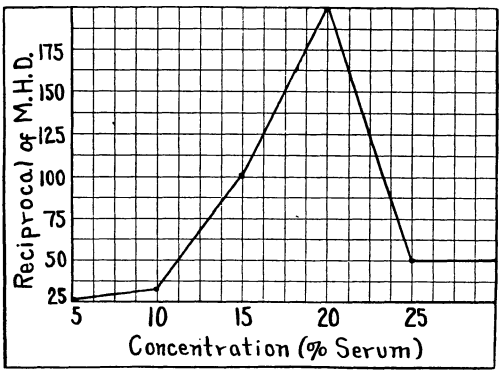

Fig. 5.—Streptolysin production in various concentrations of heated sheep serum broth, B. streptococcus, strain V.

The rise in efficiency as recorded in table 12 is strikingly presented in the curve of figure 5. The reciprocals of the MHD's are plotted on the ordinates, the concentrations of serum on the abscissae. A similar test made with beta streptococcus strain M gave values identical with those for V in concentrations of from 5 to 20%. The drop in potency occurring in 25 and 30% concentrations for V was not so clear in the case of strain M. With this strain 25% broth gave an MHD identical with that obtained at 20%, i. e., 0.005. The 30% concentration yielded a titer of 0.01.

The efficiency of heated human serum in various concentrations was next put to experiment. The concentrations studied in this experiment were those of 5, 10, 15 and 20%. The serum was inactivated at 57 C. for 45 minutes. Sheep serum, drawn on the same day as the human and heated to the same temperature was studied in parallel as control. The standard broth was used. All tubes, 8 in number, were seeded with 0.1 c c of a suspension in 4 c c of broth, of one loop of a 9-hour blood-agar culture of streptococcus M. Incubation, 8 hours in the water bath; dilutions were made and MHD determined as in all previous experiments against 0.5 c c of a 2.5% suspension of washed sheep's red blood cells. Table 13 summarizes the results of this experiment.

TABLE 13

STREPTOLYSIN IN VARIOUS CONCENTRATIONS OF HEATED SHEEP AND HUMAN SERUM BROTH

Tube	Serum Concentration		Dilutions Tested*	MHD
	Human	Sheep		
1	5	—	0.3 to 0.01	0.07
2	—	5	0.1 to 0.01	0.03
3	10	—	0.1 to 0.01	0.02
4	—	10	0.07 to 0.007	0.03
5	15	—	0.06 to 0.006	0.01
6	—	15	0.05 to 0.003	0.008
7	20	—	0.05 to 0.002	0.01
8	—	20	0.05 to 0.001	0.005

* Dilutions 0.1 to 0.01 graded by hundredths.
Dilutions 0.01 to 0.001 graded by thousandths.

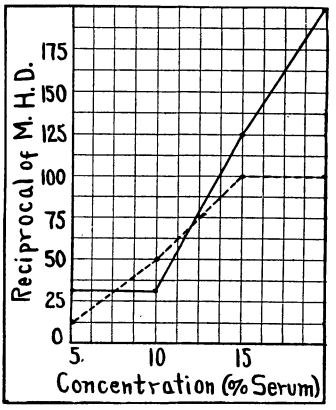

Fig. 6.—Streptolysin in various concentrations of heated sheep and human serum broth, B. streptococcus M; continuous line, sheep; broken line, human.

The results of this experiment are graphed in the curve presented in figure 6. It will be noted that for sheep serum there is very slight deviation from the curve of figure 5 which is plotted from a similar experiment with strain V. The 5% concentration shows a deviation of 0.01, the 10 and 20% concentrations give identical values while the

15% tubes show a difference of only 0.002. It will be noted further that the MHD for strain V in table 10 was 0.005 in the 20% concentration. Two other tests of 8-hour cultures in 20% sheep serum broth, using the same strain gave titers of 0.006 and 0.005, respectively. This result and the remarkable number of checks obtained in the case of strain M would indicate that under optimum conditions of test, hemolytic indices of great constancy for a given strain can be obtained. Since some investigators have made statements of an opposite nature we will return to the discussion of this question.

The experiment just recorded shows that the efficiency for hemolysin production increases directly with increase in concentration of both human and sheep serum up to at least 20%. The result of the use of higher concentrations of serum on streptolysin production will be discussed later, since we wish next to show the result of a comparative test of various concentrations of rabbit and sheep serum.

The rabbit and sheep blood used in this experiment were obtained on the same day, defibrinated, centrifugated, the serums pipetted off and heated to 57 C. for 45 minutes. Standard broth was used; 5, 10, 15 and 20% concentrations of each serum were employed. The tubes, 8 in number, were seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of a 9-hour blood-agar culture of streptococcus M. The cultures were, as usual, incubated 8 hours, at the end of this time centrifugated at 8,000 revolutions a minute, the supernatants pipetted off, iced, diluted, and graded amounts mixed with 0.5 c c of a 2.5% suspension of 3 times washed sheep's red blood cells. The final volume of all tubes was 1.5 c c. The result of the experiment is summarized in table 14.

TABLE 14

STREPTOLYSIN PRODUCTION IN VARIOUS CONCENTRATIONS OF HEATED SHEEP AND RABBIT SERUM BROTH

Tube	Serum Concentration	Dilutions Tested	MHD
1	5% Rabbit	0.2 to 0.05	>0.2
2	5% Sheep	0.2 to 0.03	0.04
3	10% Rabbit	0.2 to 0.03	0.2*
4	10% Sheep	0.1 to 0.01	0.03
5	15% Rabbit	0.2 to 0.01	0.05
6	15% Sheep	0.07 to 0.003	0.008
7	20% Rabbit	0.09 to 0.005	0.02
8	20% Sheep	0.07 to 0.001	0.005

* Complete hemolysis occurred in 0.2 c c, ++ hemolysis in 0.1 c c. No dilutions between these two figures were tested. Therefore it is possible that the real MHD may be somewhat lower than the figure recorded in the table.

The results tabulated in table 14 are plotted on the curve figure 7. The reciprocals of MHD's are plotted on the ordinates, the concentrations of serum on the abscissae. Marked differences will be seen to exist between this curve and that in figure 6. In 5% concentration of rabbit serum the largest amount of supernatant tested, 0.2 c c,

failed to give complete hemolysis. The corresponding concentration of sheep serum gave an MHD of 0.04. This figure is identical with that for the 5% concentration in table 12, and only varies by 0.01 c c from that of table 13.

In 10% concentrations there is still a very marked discrepancy in the efficiency of the two serums, rabbit recording 0.2, as compared with 0.03 for sheep. It is interesting to note here that 5 separate tests of 10% sheep serum with strain M gave an MHD of 0.03 each time. This is a good example of the manner in which titers of supernatant fluids can be quantitatively reduplicated.

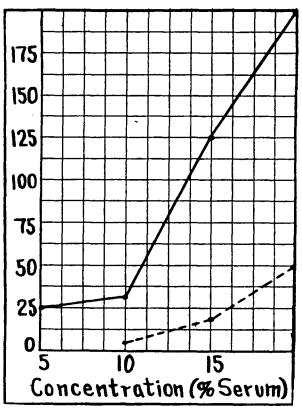

Fig. 7.—Streptolysin in various concentrations of heated sheep and rabbit serum broth, B. streptococcus M; continuous line, sheep; broken line, rabbit.

Rabbit serum, very poor in yield of streptolysin in 5% concentration and not much better in 10%, is shown by the table to be much more efficient in 15%, the MHD being in this case 0.05. The parallel tube for sheep serum exhibits an MHD of 0.008, a perfect check of the 15% concentration in table 13. Finally, 20% rabbit serum is better than 15, since an MHD of 0.02 is obtained for the former concentration. The sheep serum parallel checks that of table 13 — 0.005 c c.

It will be observed that the 20% concentrations of sheep, human and rabbit in tables 13 and 14 give titers of 0.005 in both cases for sheep and 0.01 and 0.02, respectively, for human and rabbit. In table 10 heated sheep, human and rabbit are compared in this concentration only. The same strain is used (M). The values are exactly the same, i. e., 0.005, 0.01, 0.02. This experiment was performed a month before that just recorded.

The experiment summarized in table 12 indicates that 20% serum concentration represents the optimum for streptolysin production in the case of strain V. The question arose whether this concentration was the optimum when rabbit and human serum were used, or whether the curves cross at higher concentrations.

Sheep, human, and rabbit was obtained on the same day, the serum pipetted off after centrifugation, and the serums, 24-hours old, were heated and added to the standard broth as follows:

TABLE 15

STREPTOLYSIN IN 20, 25, AND 30% HEATED HUMAN, RABBIT AND SHEEP SERUM BROTH

Tube	C C Standard Broth	Percentage	C C Serum 56 C. 45 Minutes		
			Human	Rabbit	Sheep
1	8.0	20	2.0	—	—
2	8.0	20	—	2.0	—
3	8.0	20	—	—	2.0
4	7.5	25	2.5	—	—
5	7.5	25	—	2.5	—
6	7.5	25	—	—	2.5
7	7.0	30	3.0	—	—
8	7.0	30	—	3.0	—
9	7.0	30	—	—	3.0

Tubes 1, 2, 3; 4, 5, 6; 7, 8 and 9 were seeded at 30-minute intervals. The three sets were removed from the incubating bath at similar intervals, 8 hours later. Each tube was seeded with 0.1 c c of a suspension in 4 c c of broth, of 1 loop of a 12-hour culture of streptococcus strain M. The tubes were incubated as usual for 8 hours, centrifugated at 8,000 revolutions a minute, the supernatants were pipetted off, iced, diluted with iced 0.85% NaCl solution and graded dilutions mixed with 0.5 c c of 2.5% suspension of washed sheep's red blood cells. The final volume in all tubes was 1.5 c c.

The entire series, which comprised 111 tubes, was incubated at 37 C. for 2 hours and readings taken. The results of the experiment are outlined in table 16.

TABLE 16

STREPTOLYSIN PRODUCTION IN 20, 25 AND 30% 56 C. HUMAN, RABBITT AND SHEEP SERUM BROTH

Tube	Heated Serum	Dilutions Tested*	MHD
1	Human	0.05 to 0.005	0.007
2	20% Rabbit	0.06 to 0.006	0.03
3	Sheep	0.05 to 0.003	0.005
4	Human	0.05 to 0.001	0.02
5	25% Rabbit	0.05 to 0.004	0.007
6	Sheep	0.05 to 0.003	0.005
7	Human	0.05 to 0.001	0.04
8	30% Rabbit	0.05 to 0.001	0.02
9	Sheep	0.05 to 0.001	0.01

* 0.05 to 0.01 graded by hundredths.
0.01 to 0.001 graded by thousandths.

Human serum broth shows a decided optimum at 20%, dropping from 0.007 at this concentration to 0.02 at 25% and 0.04 at 30%. Rabbit serum broth which gives an MHD of 0.03 at 20, jumps to 0.007 at 25, thus crossing the curve for human serum, but descends to 0.02 at 30%.

The MHD of 0.007 for 20% human serum, and for 25% rabbit serum represented the highest titer of streptolysin we have ever obtained from the serums of these two species.

The data summarized in table 16 are plotted in curves in figure 8.

It would be well at this point to summarize briefly the results gleaned from hemolysin production in various concentrations of heated sheep, human and rabbit serum broth. Sheep serum is distinctly superior to that of the human and rabbit in lower concentrations. It

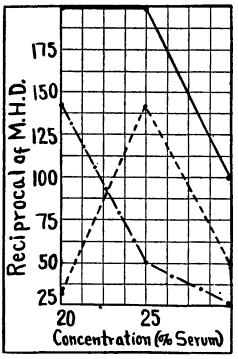

Fig. 8.—Streptolysin in 20, 25 and 30% heated human, rabbit and sheep serum broth, B. streptococcus M; continuous line, sheep; broken line, rabbit; dot and dash line, human.

reaches its optimum at from 20 to 25%, at which point it still retains its superiority over the other two species. Rabbit serum which is the poorest of the three in low concentrations rises rapidly through 15 and 20% to its optimum at 25% at which point it exhibits a titer nearly as high as that of sheep serum (0.007 as against 0.005 for sheep). Human serum, which is slightly inferior to that of sheep at 5% rises to its optimum at 20%. At higher concentrations its efficiency falls off to a point below that of rabbit and sheep.

It is important that any one of these three serums is suitable for securing streptolysin of high titer provided it is used in proper concentration. But in general, sheep serum is superior to the other two, and variation in lysin production according to concentration is not so great.

STREPTOLYSIN PRODUCTION IN FRESH UNHEATED SERUMS

It has been stated in a previous paragraph that this study of comparative quantitative hemolysin production in various concentrations of different serums had its inception in the observation of the marked

superiority of sheep over rabbit and human blood agar in demonstration of hemolytic zones. The plates used in this test were prepared with fresh defibrinated blood. This fresh unheated blood was mixed in concentration of 10% with agar. In mixtures of this blood concentration, the serum present would amount to from 4 to 6% of the total volume, depending on the proportionate volume of red blood cells to serum. It was therefore considered that comparative hemolysin production with low concentrations of fresh unheated serum would be an important subject for study. The investigation of 5% fresh unheated sheep, human, and rabbit serum broth would approximate as closely as possible the conditions present in 10% blood plates. Possible differences in amount of streptolysin produced might explain the marked difference in size of hemolytic zones, on 10% sheep as compared with rabbit and human blood plates of a similar concentration.

The subject was experimentally approached thus: Sheep, human and rabbit blood were obtained on the same day, centrifugated, the serums pipetted off, iced and used 24 hours after bleeding. Six tubes were prepared as follows:

TABLE 17

TUBES PREPARED FOR TEST

1.	9.5 c c	standard broth	+	0.5 c c	sheep serum	(5%)
2.	9.0 c c	standard broth	+	1.0 c c	sheep serum	(10%)
3.	9.5 c c	standard broth	+	0.5 c c	human serum	(5%)
4.	9.0 c c	standard broth	+	1.0 c c	human serum	(10%)
5.	9.0 c c	standard broth	+	0.5 c c	rabbit serum	(5%)
6.	9.5 c c	standard broth	+	1.0 c c	rabbit serum	(10%)

All tubes were seeded with 0.1 c c of a suspension in 4 c c of broth of 1 loop of a 14-hour culture of beta streptococcus M., incubated 8 hours at 37 C., centrifugated at 8,000 revolutions a minute for 3 minutes. The supernatants were then pipetted off, iced, diluted with iced 0.85% NaCl solution and tested in graded quantities against 0.5 c c portions of a 2.5% suspension of three times washed sheep's red blood cells. Incubation of the test mixtures, 37 C., 2 hours. Results summarized in table 18.

TABLE 18

STREPTOLYSIN PRODUCTION IN FRESH UNHEATED 5 AND 10% SHEEP, HUMAN AND RABBIT SERUM BROTH

Tube	Unheated Serum	Dilutions Tested	MHD
1	5% Sheep	0.1 to 0.01	0.06
2	10% Sheep	0.1 to 0.01	0.03
3	5% Human	0.5 to 0.02	0.2
4	10% Human	0.5 to 0.02	0.08
5	5% Rabbit	1.0 to 0.04	—
6	10% Rabbit	1.0 to 0.04	—

The results just given in this table are most instructive. Five and 10% sheep serum broth yield fairly large amounts of streptolysin, the MHD's for these concentrations being, respectively, 0.06 and 0.3. These do not differ very greatly for those of heated sheep serum in like concentration. Human serum broth in the unheated condition yielded MHD's of 0.2 and 0.08 for 5 and 10% concentrations, respectively. Both figures are much lower than those for the respective concentrations of sheep serum and distinctly lower than for similar concentrations of heated human serum. Finally, unheated rabbit serum is seen to be extremely poor for streptolysin production in 5 and 10% concentrations. No complete hemolysis was obtained in any tube, despite the fact that as much as 1 c c of undiluted supernatant was tested against 0.5 c c of washed red blood cells. Subsequent results have shown that this almost total absence of hemolysin is not met with in all tests of a similar nature. We have observed MHD's of 0.5 and 0.6 for 5% concentrations of unheated fresh rabbit serum broth. This is still extremely low as compared with the titers from like concentrations of sheep or even of human serum. It was thought important to check carefully the comparative efficiency of heated and unheated human and sheep serums, the tests to be made simultaneously from the same batches of serum. Sheep and human serums obtained on the same day and iced for 24 hours were used in this experiment.

In all experiments with unheated rabbit and human serums, controls were made to rule out the possibility of normal antisheep red cell hemolysin.

The serums were divided into two parts, one portion of each serum being heated to 57 C. for 45 minutes, while the other was left on ice. The following tubes were then prepared:

TABLE 19

TUBES PREPARED FOR TEST

1.	9.5 c c standard broth + 0.5 c c unheated human serum (5%)
2.	9.5 c c standard broth + 0.5 c c 57 C. 45 minutes human serum (5%)
3.	9.5 c c standard broth + 0.5 c c unheated sheep serum (5%)
4.	9.5 c c standard broth + 0.5 c c 57 C. 45 minutes sheep serum (5%)
5.	9.0 c c standard broth + 1.0 c c unheated human serum (10%)
6.	9.0 c c standard broth + 1.0 c c 57 C. 45 minutes human serum (10%)
7.	9.0 c c standard broth + 1.0 c c unheated sheep serum (10%)
8.	9.0 c c standard broth + 1.0 c c 57 C. 45 minutes sheep serum (10%)

These tubes were then seeded with 0.1 c c of a suspension in 4 c c of broth of 1 loop of a 10-hour blood-agar culture of streptococcus strain M. The tubes were placed for 8 hours in the water bath at 37 C., centrifugated at 8,000 revolutions a minute for 3 minutes, the supernatants pipetted off and iced and dilutions made with iced 0.85% NaCl solution. These dilutions were tested

in graded quantities against 0.5 c c of a 2.5% suspension of washed sheep's red blood cells, the final volume in all tubes being 1.5 c c. The results of the experiment are gathered together in table 20.

TABLE 20

STREPTOLYSIN PRODUCTION IN 5 AND 10% HEATED AND UNHEATED HUMAN AND SHEEP SERUM BROTH

Tube	Serum		Dilutions Tested	MHD
	Human	Sheep		
1	5% unheated	—	0.2 to 0.02	— (>0.2)
2	5% heated	—	0.2 to 0.02	0.1
3	—	5% unheated	0.1 to 0.02	0.08
4	—	5% heated	0.08 to 0.01	0.03
5	10% unheated*	—	0.1 to 0.01	0.04
6	10% heated	—	0.08 to 0.01	0.03
7	—	10% unheated	0.08 to 0.01	0.03
8	—	10% heated	0.08 to 0.01	0.03

* Controlled for human antisheep red cell hemolysin.

The results presented in table 20 confirm those recorded in table 18. For 5% active human serum, no complete hemolysis occurred when as much as 0.2 c c of supernatant was added to the red blood cells. Therefore the MHD must have been greater than 0.2. The same concentration of sheep serum gave an MHD of 0.08 (tube 3, table 20). In the case of both human and sheep the heated 5% was more favorable for lysin production than the same concentration of unheated serum. For human this difference was expressed by MHD, 0.1 as against > 0.2, for the sheep by 0.03 as against 0.08. In the 10% concentrations, both of human and sheep, this marked difference was largely obliterated. Unheated 10% human had an MHD of 0.04 as against 0.03 for the 10% heated serum. Both unheated and heated 10% sheep serum broth yielded an MHD of 0.03.

It would be well to remark that a difference as striking as this between 5% unheated human and sheep serum cannot always be demonstrated. We have observed comparative tests of a nature similar to that just recorded in which 5% unheated sheep serum broth in an 8 hour culture, the same strain, M, being used, yielded an MHD of 0.07 as compared with 0.1 for a like concentration of unheated human serum.

On the other hand, this concentration of unheated sheep serum yields pretty constantly an MHD of 0.04 to 0.08, while similar concentrations of human serum may frequently give a titer of 0.2 or > 0.2. The foregoing indicates that considerable variations occur in streptol-

ysin production in unheated sheep and human serums, when the serum is present in a small amount, but that the sheep is consistently more efficient than the human serum.

The same is true, only more strikingly so, of rabbit as compared with sheep serum. The differences in the case of rabbit serum are far greater than the ones just described for human and sheep. This is indicated clearly in the following experiment.

Twenty-four hour old rabbit and sheep serums were divided into two portions, one of each being iced and the other heated to 57 C. for 45 minutes. The following tubes were then prepared:

TABLE 21

TUBES PREPARED FOR TEST

Tube	Standard Broth	Serum Rabbit		Serum Sheep	
		Unheated	57 C. 45 Minutes	Unheated	57 C. 45 Minutes
1	9.5	0.5	—	—	—
2	9.5	—	0.5	—	—
3	9.5	—	—	0.5	—
4	9.5	—	—	—	0.5

These tubes were seeded with 0.1 c c of a suspension in 4 c c of broth of 1 loop of a 9-hour culture of beta streptococcus M, incubated for 8 hours at 37 C., centrifugated at 8,000 revolutions a minute for 3 minutes, the supernatants iced, diluted and tested against 0.5 c c of a 2.5% suspension of 3 times washed sheep's red blood cells.

The results of this experiment are presented in table 22.

TABLE 22

STREPTOLYSIN PRODUCTION IN 5% HEATED AND UNHEATED RABBITT AND SHEEP SERUM BROTH CULTURES

Tube 1 (5% Unheated Rabbit Serum)			Tube 3 (5% Unheated Sheep Serum)			Tube 2 (5% 57 C. 45 Minutes Rabbit Serum)			Tube 4 (5% 57 C. 45 Minutes Sheep Serum)		
Amount Supernatant	Result	MHD	Amount Supernatant	Result	MHD	Amount Supernatant	Result	MHD	Amount Supernatant	Result	MHD
1.0	C		1.0	C		1.0	C		0.1	C	
0.75	C		0.09	C	0.09	0.75	C		0.09	C	
0.5	C	0.5				0.5	C		0.08	C	0.08
			0.08	C—		0.3	C				
0.3	C—		0.07	++		0.2	C		0.07	C—	
0.2	++		0.06	+		0.18	C	0.18	0.06	++	
0.15	+		0.05	+					0.05	+	
0.1	tr.		0.04	tr.		0.16	C—		0.04	+	
			0.03	0		0.16	C—		0.03	tr.	
			0.02	0		0.12	C—		0.02	tr.	
			0.01	0		0.1	+		0.01	0	

The experiment just summarized confirms the quantitative relations indicated in tubes 1 and 2, 5 and 6, table 18. In the present experiment, however, the difference is not quite so marked, since the rabbit serum (unheated) shows a higher titer than in table 18, and the unheated sheep serum of this experiment is slightly lower in lysin content than that of the earlier test. The central fact is, nevertheless, clearly checked. Unheated sheep serum in low concentration is far superior to unheated rabbit serum for the production of streptolysin.

Higher concentrations of all three of the serums just studied have been investigated in regard to hemolysin production. Twenty per cent. heated and unheated sheep, rabbit and human serum broth was seeded with the usual amount of a 20-hour culture of beta streptococcus M. Test of the supernatants, after 8 hours of incubation at 37 C., gave the results summarized in table 23.

TABLE 23

STREPTOLYSIN PRODUCTION IN HEATED AND UNHEATED 20% SHEEP, HUMAN AND RABBIT SERUM BROTH

Tube	Serum, 20%			MHD
	Sheep	Human	Rabbit	
1	Unheated	—	—	0.01
2	56 C. 45 minutes	—	—	0.01
3	—	Unheated	—	0.02
4	—	56 C. 45 minutes	—	0.02
5	—	—	Unheated	>0.05*
6	—	—	56 C. 45 minutes	0.05

* Lower dilutions not made; ++ hemolysis in 0.05; MHD not obtained.

From this experiment one might be led to conclude that in 20% concentration no perceptible difference in efficiency exists between heated and unheated sheep, or heated and unheated human serum. For 20% rabbit serum heated is apparently somewhat superior to unheated serum, although the failure to use a dose higher than 0.05 c c made a strict quantitative comparison impossible in this experiment. It is quite probable that the low titer of lysin obtained in all cases is due to the use of a 20-hour culture for seeding. In cases of this kind the organisms transferred to the serum broth tubes would in all probability exhibit a longer period of lag. Consequently, the point of maximum production of streptolysin would have occurred at a time later than that at which incubation was cut off (8 hours). The experiment summarized in table 6 lays stress on the importance of the use of really young cultures for seeding when it is desired to secure an early peak of hemolysin content.

Similar experiments were made with fresh unheated horse serum, very kindly supplied to us by the research department of Parke, Davis & Co. The first experiment with this material concerned itself with the quantitative study of lysin produced in 5, 10, 15 and 20% concentrations of fresh unheated horse serum broth.

TABLE 24

TUBES PREPARED FOR TEST

1.	9.5 c c	standard broth	+	0.5 c c	unheated horse serum	(5%)
2.	9.0 c c	standard broth	+	1.0 c c	unheated horse serum	(10%)
3.	8.5 c c	standard broth	+	1.5 c c	unheated horse serum	(15%)
4.	8.0 c c	standard broth	+	2.0 c c	unheated horse serum	(20%)

All tubes were seeded with 0.1 c c of a suspension in 4 c c of broth of 1 loop of a 10-hour culture of beta streptococcus M. They were incubated for 8 hours at 37 C., centrifugated at 8,000 revolutions a minute for 3 minutes, the supernatants from this centrifugation iced, diluted with iced 0.85% NaCl; tested against 0.5 c c of a 2.5% suspension of washed sheep's red blood cells. Final volume in all tubes 1.5 c c. The results are given in table 24.

TABLE 25

STREPTOLYSIN PRODUCTION IN 5, 10, 15 AND 20% 24-HOUR, UNHEATED HORSE SERUM BROTH

Tube	Horse Serum, Percentage	Dilutions Tested*	MHD	Hemolytic Index
1	5	0.2 to 0.01	0.06	16.6
2	10	0.1 to 0.01	0.02	50.0
3	15	0.05 to 0.003	0.01	100.0
4	20	0.05 to 0.001	0.007	142.8

* From 0.2 to 0.1 dilutions graded by two-hundredths.
From 0.1 to 0.01 dilutions graded by hundredths.
From 0.01 to 0.001 dilutions graded by thousandths.

The results of table 25 indicate a very close correspondence in the amount of hemolysin produced between fresh unheated horse and fresh unheated sheep serum. This will be seen by comparing the results just summarized for horse serum with those for unheated sheep serum in tables 18 and 20.

It is unfortunate that no comparative test was made at this time of fresh unheated and heated horse serum broth, in 20% concentration. The same serum used in the experiment just described but aged for several days and heated to 56 C. for 45 minutes, gave an extremely potent lysin, the MHD reaching 0.003 in two instances. On the other hand, the 20% concentration of unheated serum (tube 4), table 25, gave an MHD of 0.007. This discrepancy was considered

sufficiently wide to justify a parallel test of heated and unheated horse serum in the concentration of 20%. It was also attempted at this time to recheck the unheated 5% concentration.

The serum used in this test was 72 hours old, consequently the results cannot be strictly compared with those obtained from the experiment in which 24-hour serum was used. The findings observed are brought together in table 26.

TABLE 26

STREPTOLYSIN PRODUCTION IN 5 AND 20% UNHEATED AND 20% HEATED HORSE SERUM BROTH

Tube	Serum Percentage		Dilutions Tested*	MHD
	Unheated	56 C. 45 Minutes		
1	5	—	1.0 to 0.01	0.03
2	20	—	0.1 to 0.001	0.01
3	—	20	0.1 to 0.001	0.006

* Dilution 0.1 to 0.01 graded by hundredths.
Dilutions 0.01 to 0.001 graded by thousandths.

It will be observed (tube 1) that the MHD obtained for the 5% concentration was 0.03. This figure is 0.03 higher than that of the corresponding dilution in table 25. The serum used in the experiment just described was 72 hours old, as against 24 hours for that employed in table 25. Hence no strict comparison can be drawn. It is probable that the difference in age of serum is responsible for this difference in result. For it is quite likely that suitability of serum for hemolysin production might be increased by aging as well as by heat. This is true for other properties of serum. For example, heat of 56 C. and aging both have a deleterious effect on the alexic and the "serozyme" functions of serum.

Tubes 2 and 3, table 26, show a perceptible difference in titer. This would indicate a greater suitability for hemolysin production of heated horse serum in 20% concentrations. The result given in table 23 would indicate that this was not the case for similar concentrations of unheated sheep and human serum. It is true, on the other hand, that the comparative tests of heated and unheated human and sheep serum in this concentration were not exhaustive enough to allow one to draw sweeping conclusions. It may be remarked, too, that the difference in the case of horse serum, while perceptible, is not striking, since it shows 0.01 for the unheated, 0.006 for the heated serum.

It would be logical at this place to discuss the comparative value of horse and sheep serum broth for streptolysin production. Heated horse serum in 20% concentration has given in some instances MHD's higher than the best titer obtained with like amounts of heated sheep serum. Beta streptococcus, strain M, was used in all cases now under discussion. At the same time, it has been more difficult to obtain with horse serum the strikingly constant results observed in 20% heated sheep serum for strain M. The large number of times an MHD of 0.005 was obtained in this medium for strain M will be recalled. In similar concentrations of horse serum the MHD for this strain varied from 0.003 to 0.007.

Von Hellens[7] states that maximal hemolysin production occurs in concentrations of 30 and 40% horse serum broth. M'Leod[5] and M'Leod and McNee[6] assert that 15 to 20% is the optimum concentration. The following experiment strongly supports the latter opinion.

The usual amount of an 8-hour culture of streptococcus M was seeded into tubes, labeled 1, 2 and 3, containing respectively 20, 30 and 40% of heated horse serum broth. The broth was of the standard type used in all previous experiments. The usual 8-hour incubation, centrifugation at 8,000 revolutions a minute, and the dilution was carried out and tests were made of dilutions graded from 0.05 to 0.001 against 0.5 c c of a 2.5% suspension of sheep's red blood cell suspension. The final volume in all tubes was 1.5 c c; incubation, 2 hours at 37 C. The results are given in table 27.

TABLE 27

STREPTOLYSIN PRODUCTION IN 20, 30 AND 40% HORSE SERUM BROTH

Amount Supernatant	Horse Serum 56 C. 45 Minutes					
	20%		30%		40%	
	Result	MHD	Result	MHD	Result	MHD
0.05	C		C		C	
0.04	C		C		C	
0.03	C		C		C	
0.02	C		C		C	
0.01	C		C		C	
0.009	C		C		C	
0.008	C		C		C	
0.007	C		C		C	
0.006	C		C		C	
0.005	C		C		C	
0.004	C		C		C	
0.003	C	0.003	C	0.003	C	0.003
0.002	++		C—		C—	
0.001	+		++		++	

The striking reduplication of MHD occurring in all three concentrations does not argue for a marked superiority of the 30 and 40% over the 20% concentrations.

DISCUSSION OF RESULTS OBTAINED WITH VARIOUS CONCENTRATIONS OF UNHEATED AND HEATED SHEEP, HUMAN, RABBIT, AND HORSE SERUM

In all of the experiments described, eight-hour incubation was used. Beta streptococcus, strain M, was employed in all but a few instances. In these few the studies were conducted with strain V, which has a hemolytic index practically identical with that of M.

For all serums studied, heated serum, especially in the lower concentrations, yields better lysin than unheated fresh serum. This difference between heated and unheated serum is quantitatively most striking in a 5% concentration of rabbit serum. It is marked in 5% concentration of human serum; less so, but still perceptible in like amounts of sheep serum.

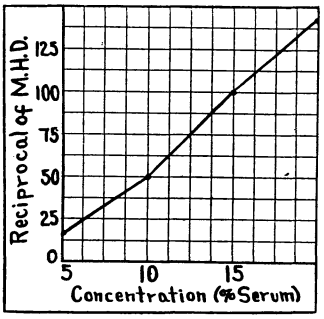

Fig. 9.—Streptolysin production in various concentrations of unheated horse serum broth, B. streptococcus M.

This difference in efficiency for streptolysin production becomes less as higher concentrations are reached. In 20% concentrations there is still a shade of superiority of heated over unheated horse and rabbit serum. Although the investigation of this point was not exhaustive, practically no divergence of titer is to be found in 20% concentration of sheep or human serum.

All serums, active or inactive, rise sharply from comparatively low titers in 5% concentration to high values which reach their maximum at 20% for horse, sheep and human, at 25% for rabbit. The curves for unheated horse and human serum, plotted in terms of MHD on the ordinates and concentrations of serum on the abscissae, have less steep slopes from low to high concentrations than those of human and rabbit (see figure 10). This means that the actual amount of hemolysin in unheated human and rabbit serum is far less in low con-

centrations than that produced in similar concentrations of sheep and horse. Study of the curves in figure 10 will make this clear. These curves indicate the actual amount of lysin present and not the proportionality expressed when reciprocals of the MHD are plotted. It will be seen that in the low concentrations, human and rabbit are far apart from sheep and horse. As the concentrations increase, the curves converge, which illustrates well the fact that all of the serums are fairly efficient for lysin production at a certain concentration. This explains, we believe, the marked difference in size of the hemolytic zones produced by beta streptococcus on horse and sheep as compared with human and rabbit 10% blood-agar plates, although in the case of human blood-agar plates another factor operates. This factor is discussed in the second part of this paper.

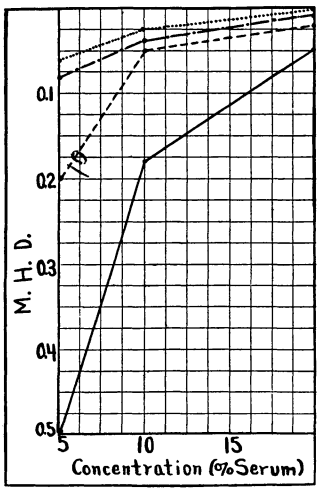

Fig. 10.—Comparative streptolysin production in 5, 10 and 20 concentrations of unheated horse, sheep, human and rabbit serum broth, B. streptococcus M; dotted line, horse; dot and dash line, sheep; broken line, human; continuous line, rabbit.

PART II

A NEW BLOOD-AGAR PLATE, DEVISED FROM THE DATA OBTAINED IN PART I

Holman[3] points out the necessity of a medium of maximal differentiating power for the fermentation reactions of streptococci. Brown[2] stresses a similar need in regard to blood plates as indicators of hemolysin and green production. He follows this demand by the description of a medium admirably suited to this purpose, i. e., horse blood

agar. At the same time it would seem that the data obtained in Part 1 of this paper could be utilized in the synthesis of a plate which might present still further advantages, without introducing a too complicated technic.

Before describing the plate which has been devised on the basis of the experiments just mentioned, certain observations with the ordinary 10% horse, sheep, human and rabbit blood-agar plates will be recorded. These experiments, with very few exceptions, were carried out with poured plates. That is, dilute suspensions of beta streptococcus were transferred by platinum loop to liquid mixtures of blood and agar, the tubes thoroughly shaken to effect even mixture of the blood, agar and bacteria, and the plates immediately poured. The advantages of this procedure over the more facile streak method are fully explained by Blake[4] and by Brown,[2] and need not be dwelt on in this place.

The nutrient agar used for blood plates was made by adding agar in 1.5% concentration to the standard broth described in Part I. Reaction of broth P_H 7.6-7.8, Parke, Davis & Co., bacteriologic peptone, 2%. Very recently we have found that a lower alkalinity is perhaps more suitable. Excellent results can be obtained with broth to which 16 c c per liter of N Na_2CO_3 are added.

Large test tubes containing 8 c c of this agar are liquefied, cooled to 45 C., and 0.8 c c of defibrinated blood added. The tubes are then seeded with streptococci, the blood and agar thoroughly mixed, and poured at once into flat-bottomed Petri dishes, 9 cm. in diameter. The plates are allowed to harden, and are then incubated in an inverted position.

The hemolytic zones surrounding the colonies on sheep blood plates are considerably larger, are very much more clear, and have more cleanly scalloped margins than those on parallel plates of human and rabbit blood. The results of an experiment with beta hemolytic streptococcus M on parallel 10% sheep, human and rabbit blood-agar plates are summarized in table 28.

TABLE 28

DIAMETER OF HEMLYTIC ZONES ON 10% SHEEP, HUMAN AND RABBIT BLOOD AGAR

Plate	Kind of Blood	Percentage	Length of Incubation	Average Diameter of Hemolytic Zones in Millimeters
1	Sheep	10	14 hours	2.3
2	Human	10	14 hours	1.53
3	Rabbit	10	14 hours	1.5

Brown[2] notes that zones occurring on human blood-agar plates are cloudy, with indefinite borders. He does not think that this condition is due to unhemolyzed cells. In this opinion we differ with Brown. Microscopic examination of the zones surrounding colonies of beta streptococcus on human blood agar reveal residual, perfectly distinct clumps of unhemolyzed red blood cells. What is more, the vague, indefinite, fringed border of the zones is due to the same cause. This persistence of unhemolyzed clumps is undoubtedly the result of the strong hemagglutination occurring when human blood is mixed with agar in certain proportions. No matter how carefully and thoroughly these are mixed, microscopic examination of a poured plate reveals large numbers of small, closely packed clumps of red blood cells. It is probably the failure of the streptolysin to penetrate completely into these clumps that results in the incomplete laking of these masses, so giving the milky indefinite appearance to the hemolytic zones.

This observation led us to study quantitatively the hemagglutinative action of agar on the blood of different species. The method used was to mix decreasing quantities of 1.5% agar with constant amounts of the whole blood of various species. All tubes were brought to constant volume with 0.85% NaCl solution and the mixtures incubated at 45 C. While the quantitative amounts of agar able to cause agglutination of the different bloods did not vary widely, there was a marked difference in the speed and intensity of the reaction for different ones. Thus, while the smallest amount of agar able to produce agglutination might be approximately the same for all the kinds of blood tested, the reaction took place far more rapidly and completely in human blood than in the others.

What is more, the clumping of cells, once established, was much less easily reversible for human blood than for sheep or rabbit. When hemagglutination takes place in agar sheep blood mixtures, the cells can be brought to homogeneous resuspension by gentle tapping of the tube, and the subsequent reagglutination is slow. It is more difficult to break up the clumps of human cells, and reagglutination takes place much more rapidly. It will be understood that this fact makes it difficult to secure a human blood plate in which the cells are homogeneously distributed. The whole reaction takes place in a very few minutes, where blood plates are concerned, since the setting of the agar after pouring terminates the agglutination reaction.

Rabbit blood occupies a position midway between that of sheep and human in regard to agglutination by agar. Sheep blood agglutinates by far the most slowly of any of the three, with a resulting greater homogeneity of the blood-agar mixture.

Horse blood, as well as the others mentioned, is agglutinated by agar, although not with the intensity or rapidity of that of human. It must be remembered also, that the large amounts of streptolysin produced by streptococci, even in low concentrations of horse blood, as compared to the smaller amounts produced in human, are better able to penetrate and hemolyze the clumps that are formed.

The difference in size of the hemolytic zones on sheep, human, and rabbit blood-agar plate is explicable by consulting figure 10. The amount of hemolysin secreted in 5% concentrations of the different serums will be noted. Human serum occupies a position midway between the highly efficient sheep and horse serum and the unsuitable rabbit serum. In 10% blood plates, more lysin is doubtless manufactured from human blood than from that of the rabbit. Human blood is made less desirable, however, by the unevenness, due to hemagglutination, of the agar-blood mixtures.

To sum up, horse and sheep blood are distinctly superior to rabbit and human blood plates. The hemolytic zones produced by beta streptococcus on the former media are larger, clearer, and have more sharply defined borders. Human blood is inferior to sheep; first, because considerably less hemolysin is produced, in low concentrations used in plates; second, because of the energetic agglutination of human red blood cells when mixed with agar. The borders of the zones on human blood agar are so irregular that it is difficult to measure accurately the diameter of the hemolyzed area.

While hemagglutination is not a factor of importance in rabbit blood-agar plates, the small amount of lysin produced in low concentrations of rabbit blood causes hemolytic zones much inferior in size to those of sheep or horse blood. The zones on sheep and horse blood in plates with similar concentration of blood, using the same organism, are very nearly of the same size and quality. Slightly more lysin may be produced in these concentrations of horse blood. This is counterbalanced by the greater homogeneity of the sheep blood-agar mixtures. The suitability of sheep blood has been stressed only because it is more accessible to general use than horse blood.

A BLOOD PLATE GIVING MAXIMAL STREPTOLYSIN PRODUCTION

Analysis of the data obtained in Part 1 of this paper resulted in the synthesis of a blood-agar plate which should satisfy the demand for a medium of maximum differentiating power. Sheep blood was used in the majority of these experiments. It has the advantages of being easily obtainable, only slightly agglutinable by agar, and being highly potent as a pabulum for lysin production.

Taking advantage of the observation that maximal streptolysin production occurs in 20% concentrations of heated sheep serum (figures 5, 6, 7 and 8; Part 1), a plate was devised to contain serum in this amount. It will be obvious that if whole blood were used, a concentration of at least 40% would have to be employed to reach this amount of serum. If so much blood were used, the additional number of red blood cells added would offset the increased streptolysin produced in such serum concentration. Consequently, serum-blood cell mixtures were made. The red blood cells were combined with heated serum in such proportion that when mixed with the agar the final concentration of serum would be 20%; cells, 5%; agar, 75%.

This procedure is accomplished without great technical difficulty, and an example of the exact technic employed is given below.

Sheep blood is obtained, defibrinated carefully, centrifugated, the serum pipetted off aseptically and heated to 56 C. for 45 minutes. This serum is then recombined with the red blood cells in the proportion of four parts of serum to one part of cells. One part of this serum-cell mixture is then added to three parts of liquid agar, cooled to 45 C., the mixture seeded, the tube thoroughly shaken to secure even distribution of the agar, blood, and bacteria, and its contents immediately poured into plates.

It is desirable to ice the plates so made for some minutes previous to incubation, since the relatively large amount of the serum-cell mixture causes the agar to set rather slowly. Once set, these plates hold up perfectly, even in an inverted position.

To obtain good results it is necessary always to mix the cells and serum before addition of the agar. Red blood cells added alone are energetically agglutinated by agar. On the other hand, the preponderance of serum in the serum-cell mixture seems to protect the cells from agglutination, and perfect mixture can be secured.

The sheep serum-cell mixture plate just described is the most suitable of the plates we have studied, for the rapid diagnosis and

differentiation of beta hemolytic streptococci. The organisms grow rapidly and abundantly on this medium, a maximal amount of hemolysin produced gives rise to large and clean-cut hemolytic zones, and the mixture of the blood and agar is homogeneous, provided proper precautions are taken. Table 28 gives some indication of the efficiency of this type of plate, compared to the ordinary 10% sheep, human and rabbit blood medium.

The technic used in this experiment was identical with that just described. Blood from all sources was obtained on the same day. Beta streptococcus, strain M, was used for seeding the plates.

TABLE 29

DIAMETER OF HEMOLYTIC ZONES ON 25% SHEEP SERUM-CELL MIXTURE PLATES AND ON 10% SHEEP, HUMAN AND RABBITT BLOOD-AGAR PLATES

Plate	Medium	Hours of Incubation	Diameter of Zones in Millimeters
1	25% sheep serum-cell	14	3.8
2	10% sheep blood	14	2.3
3	10% rabbit blood	14	1.3
4	10% human blood	14	1.5

Nine hours after seeding, the zones on Plate 1 had reached a diameter of 2.9 mm. Similar experiments with other strains of beta streptococcus show a like superiority of the special medium just described. If young cultures (8-12 hours) are used for the seeding of plates, diagnosis can be made at a very early period of incubation (5 to 7 hours).

The figures in table 29 represent the total diameter of the hemolytic zone, including the colony. It must not be inferred that this result represents the widest zone obtained. With heavy growing surface colonies, we have observed zones which attained diameters of 10 to 12 mm. in 24 hours. The deep colonies on such plates were, of course, much smaller, but the zones surrounding them measured in some cases 6 mm.

It is obvious that any of the four bloods studied can be treated similarly to that just described for sheep blood. In the case of horse and human blood the cell serum mixture which would be most desirable is identical with that just described (see figures 6, 8 and 9; Part I). For rabbit blood a still higher concentration of serum represents the optimum. In this instance 25% serum should be used.

While all of these bloods, combined in the manner described above, are far superior to 10% blood plates, sheep serum-blood cell mixture

is especially recommended for a standard plate, for the reasons repeatedly mentioned in previous pages.

To sum up, an attempt has been made to devise a blood plate which will contain the optimum amount of serum necessary for maximum lysin production, and at the same time the number of red blood cells approximately equal to that of an ordinary 10% plate. To effect this purpose, artificial serum blood cell mixtures are made. This is done by centrifugating the blood, removing the supernatant serum, heating it to 56 C. for 45 minutes, and recombining this heated serum with the blood cells in the proportion of 4 parts of serum to 1 part of cells in sheep, horse and human blood. For rabbit blood the proportion of serum to cells would be 5 to 1.

Such serum cell mixtures are to be added to liquid agar in the proportion of 25 to 75 of agar for the first three kinds of blood, in proportion of 30 to 70 of agar for rabbit blood.

It may be remarked here that the factor of hemagglutination, so perceptible in the ordinary 10% human blood-agar plate, is much reduced in the human serum-cell mixture medium. The larger proportion of serum present in the latter would seem to exert some protective effect on the ability of agar to clump human red blood cells.

ALPHA-HEMOLYTIC STREPTOCOCCUS ON SHEEP SERUM-CELL MIXTURE PLATES

The formation of concentric rings of alternate methemoglobinized and hemolyzed corpuscles around colonies of alpha hemolytic streptococci is a phenomenon first observed by Konrad,[15] Sigwart[16] and Saito,[17] and afterward thoroughly studied by Brown.[2] The last named investigator came to the conclusion that this effect is due to the subjection of plates containing the alpha streptococcus to alternations of incubator and room or cold room temperatures. By resorting to successive incubation and icing, each interval lasting 48 hours, Brown was able to produce several concentric rings.

Immediately round the colony lies a zone of green unhemolyzed corpuscles, which is in turn surrounded by a hemolyzed area, appear-

[15] Beitr. Geburtsh. u. Gynäk., 1908, 9, 13, p. 364.
[16] Münch. Med. Wchnschr., 1909, 56, p. 1128.
[17] Arch. Hyg. u. Infektionskr., 1912, 75, p. 121.

ing when the plate is iced after the first incubation. Subsequent incubation results in a green zone outside of the hemolyzed one, which may be again surrounded by a hemolyzed area if one resorts to a second chilling of the plate.

We have studied this property of zone formation with a strain of alpha hemolytic streptococcus (viridans), on 10% rabbit, human and sheep blood plates, and compared the results with these mediums with those secured on the sheep serum-cell mixture described above. The concentric ring picture presented by this organism on the latter medium is a striking one and much superior to that on the ordinary 10% blood agar. Of the 10% blood-agar plates, human blood furnished the poorest medium for the demonstration of ring formation; rabbit blood presented a fair picture. Ten per cent. sheep blood agar was efficient, but far less so than the sheep serum cell mixture plate.

Twenty-four hours suffice for each period of successive incubation and icing when the special medium is used. The hemolytic zones are very clear, and the bands are sharply delineated, so that they can be easily distinguished by the unaided eye. The ring formation is much more marked in the deep than in the surface colonies. This alone forms a potent argument for pouring, rather than the surface streaking of plates.

There is no doubt that the investigations of Brown have changed materially the method of differentiation of green producing and hemolytic streptococci, since this worker has definitely shown that green production is not the sole thing to be looked for on blood plates seeded with viridans or pneumococcus. Given proper conditions hemolysis also makes its appearance.

The 25% sheep serum-blood cell mixture plate furnishes optimum conditions for hemolysin production by the beta hemolytic streptococcus. For the alpha hemolytic organisms, viridans and pneumococcus, the ring effect is striking and clean cut, it being possible to produce with ease quintuple zones by the use of this medium. Up to the present time this plate seems to us to satisfy better than any other the demand for a medium of maximal differentiating power for the different varieties of streptococcus.

Unfortunately, we have not had access to any strains of the alpha prime type described by Brown. It is this type and the beta types of

very low hemolytic power that the serum-blood cell mixture plates will be of greatest use in picking up. It is well known that various strains of the beta type vary greatly in the amount of their hemolysin production. Those producing very small amounts, which might on 10% rabbit or human blood agar be confused with alpha prime or gamma would be exposed to optimum conditions for hemolysin production on the medium we propose.

It has not been possible for us to judge as to the relative merits of the blood plate and the blood broth medium in clinical investigations. The method of Lyall,[14] for the diagnosis of hemolytic streptococcus is at best qualitative and does not furnish adequate differentiation from the green producers. Throat cultures and study of the lungs and other organs at necropsy are best carried out in blood plates. For isolation from the blood stream it would seem best to utilize serum broth medium, following this by subplating at the moment cocci are demonstrated in the broth culture.

We have no opinion on the relation of hemolytic power of beta streptococcus to its virulence, and we do not think that this moot point has been satisfactorily settled. If this is to be done, liquid medium (serum broth) must be employed, since the evidence furnished by plates is qualitative only. In the employment of such serum broth for quantitative estimation of hemolytic power certain conditions should be secured. The data presented in Part I delineate quite clearly those conditions.

SUMMARY AND CONCLUSIONS

Hemolysin production in serum broth by beta hemolytic streptococcus has been studied. The amount of free hemolysin present at different intervals of incubation has been determined by the titration of the supernatants of high speed (8,000 revolutions a minute) centrifugation of serum broth cultures against constant quantities of washed red blood cells.

This method presents superiorities to those of titration of filtrates or of whole culture. These advantages are discussed in Part I.

Lysin production reaches its maximum for the strains studied at a very early period in the life of the serum broth culture. When the serum broth under test is seeded with young (8-12 hour) cultures, this

peak is reached at from 7 to 8 hours. When older cultures are used for seeding, the crest may be deferred to 12 hours.

The outpouring of streptolysin occurs during the early part of the logarithmic growth period of the culture. When this growth period ceases, the free lysin begins to decrease rapidly, often disappearing completely in 14 hours.

When streptolysin has disappeared completely from the supernatants of high speed centrifugation (free lysin) whole cultures may still be hemolytically active in low titer. This activity is probably due to lysin operating from the surfaces of organisms, at which place it is destroyed less rapidly than in the free state.

These observations pave the way for accurate quantitative titration of the streptolysin produced by a given strain of beta streptococcus. One strain, tested frequently over a period covering several months, yielded remarkably constant titers of hemolysin.

The comparative efficiency of sheep, horse, human and rabbit serum broth for hemolysin production has been studied. Heated serums are in general superior to unheated. Sheep and horse serum are distinctly superior in all concentrations to human and far superior to rabbit serum broth. Curves of the relation of serum concentration to hemolysin production have been plotted and the optimum concentration for the various serums obtained.

The data gleaned in these quantitative studies have been used in the synthesis of a new blood-agar plate. This plate has been devised with the idea of using the optimum serum concentration for maximal lysin production of a blood which shall be efficient and at the same time accessible to general use.

Sheep blood is believed to fill this need. To obtain maximal lysin production rather high concentrations of serum are necessary (20%). The number of red blood cells is kept down by making artificial combinations of heated serum and cells.

The serum-cell mixture is made by combining 4 parts of heated serum with 1 part of cells. This mixture is then combined with liquid agar in proportion of 1 part of the mixture to 3 of agar. The final concentration therefore is: serum, 20%; cells, 5%, and agar 75%.

Rabbit and human serums can also be combined with their respective cells in a similar manner, the proportions being altered slightly in the case of rabbit serum. Plates made from these bloods, while

superior to the ordinary 10% blood plates, are not as efficient as the sheep serum-cell mixture plate just described, for reasons given in Part II.

The proposed medium is not only a very suitable one for demonstration of the hemolytic zones of beta streptococcus, but is in addition very efficient in the demonstration of multiple concentric zones of green production and hemolysis for alpha streptococcus and pneumococcus.